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Supporting Information

Mulligan *et al.* 10.1073/pnas.0809979106

SI Methods

Construction of Low-Copy Vectors Expressing *nanT* and *siaPQM*. To express the *N*-acetylneuraminic acid (Neu5Ac) transporter genes *in trans*, the *nanT* and *siaPQM* genes were amplified from genomic DNA of *E. coli* MG1655 and *H. influenzae* Rd KW2, respectively, using KOD Hot Start Polymerase (EMD Biosciences), and using oligos *nanT*-Asp718G and *nanT*-BamHI for *nanT*, and *siaPQM*-Asp719G and *siaPQM*-BamHI for *siaPQM* (see supporting information (SI) Table S1); these PCR products were cloned into the low copy-number vector pWKS30 (1) resulting in constructs pES1G (*nanT*) and pES7 (*siaPQM*).

Expression and Purification of SiaP and VC1779. SiaP was cloned into pBADnLIC to create a decahistidine tagged version (pBADnP). VC1779 was amplified by using primers pRY11F and pRY11R and cloned into pET20b. The resulting plasmid pRY11, expresses VC1779 with the leader sequence encoded by the *pelB* gene replacing its native signal sequence and contains a C-terminal hexahistidine tag.

For expression of ligand-free SiaP, MC1061/pBADnP was grown at 37°C in M9 minimal medium supplemented with 100 µg/ml ampicillin, 0.2% (wt/vol) glycerol, 40 µg/ml L-leucine, 40 µg/ml L-isoleucine, 50 µg/ml 4-methyl-5-β-hydroxyethylthiazole (THZ), 2 µg/ml thiamine, 50 µg/ml nicotinic acid, and 1 mM MgSO₄ (2), to an A₆₅₀ of 1, at which point they were induced with 5 × 10⁻³ % L-arabinose. Cells were harvested after 2 h growth by centrifugation at 4430 × *g* for 15 min at 4°C and were then resuspended in 50 mM potassium phosphate, pH 7.8, supplemented with 20% (wt/vol) glycerol. VC1779 was expressed in *E. coli* BL21(DE3) pLysS in M9 minimal medium with 100 µg/ml ampicillin and 30 µg/ml chloramphenicol. Cells were grown and protein prepared from periplasmic fractions as reported (3), except that only 0.5 mM IPTG was used for overnight induction at 25°C. For Western blot analysis, we used an anti-tetraHis antibody (Sigma).

SiaP was purified in a two-step procedure: The first step using Ni²⁺-NTA superflow resin and elution of the protein in WB containing 500 mM imidazole. After dialyzing the eluted protein against 20 mM Tris-HCl, pH 8.0, the second step involved DEAE Sepharose (Sigma) anion-exchange chromatography. The protein was eluted from the DEAE resin at 125 mM NaCl by using a 0–500 mM NaCl gradient. VC1779 was purified in a single step, by using a His-TRAP column (GE Healthcare) and eluting with 250 mM imidazole. Both proteins were dialyzed overnight at 4°C into 50 mM potassium phosphate pH 7.0 before storage at –80°C or use in the transport or ligand-binding assays.

Membrane Vesicle Preparation. The SiaQM-containing membrane vesicles were prepared by rupturing the cells in at least 2 passes through a French pressure cell (Thermo) at 10,000 psi. The cells were present in 50 mM potassium phosphate, pH 7.8, 20% (wt/vol) glycerol supplemented with 100 µg/ml DNase I (Sigma) plus 1 mM MgCl₂, and, after cell lysis, the mixture was incubated on ice for 5 min with 5 mM EDTA, pH 7. Unbroken cells and cell debris were removed by centrifugation at 10,000 × *g* for 20 min. The resulting supernatant was then centrifuged at 200,000 × *g* for 1 h at 4°C and the membrane pellets were resuspended in 50 mM potassium phosphate, pH 7.0, snap-frozen in liquid nitrogen, and stored at –80°C. *L. lactis* membrane vesicles were prepared as described in ref. 4.

Purification of SiaQM. Membrane vesicles containing SiaQM were resuspended to 3.33 mg/ml protein and solubilized in 50 mM potassium phosphate, pH 7.8, 20% (wt/vol) glycerol, 200 mM NaCl, 0.5% (wt/vol) *n*-dodecyl-β-D-maltoside (DDM), and 10 mM imidazole, pH 7.8, by inversion mixing and incubated on ice for 30 min. Insoluble material was removed by centrifugation at 200,000 × *g* for 20 min at 4°C. The supernatant was incubated with Ni²⁺-NTA superflow resin (Qiagen, 0.1 ml/10 mg of protein; pre-equilibrated in 50 mM potassium phosphate, pH 7.8, 20% glycerol, and 200 mM NaCl (WB buffer) containing 0.04% DDM and 10 mM imidazole for 1 h in disposable polystyrene columns (Pierce). The resin was washed with 10 column volumes of WB supplemented with 0.04% DDM (wt/vol) and 40 mM imidazole, followed by 10 column volumes of WB with 0.1% *n*-decyl-β-D-maltoside (DM) (wt/vol) and 40 mM imidazole. SiaQM was eluted with elution buffer [WB plus 0.1% DM (wt/vol) and 500 mM imidazole]. Five mM EDTA was added to the elution fractions. SiaP and VC1779 were purified by using nickel-affinity and anion-exchange chromatography (see [SI Methods](#)).

Membrane Reconstitution of SiaQM. Liposomes were prepared from a 3:1 (wt/wt) ratio of purified *E. coli* lipids and L-α-phosphatidylcholine from egg yolk (Avanti Polar Lipids). A volume of purified SiaQM containing 200 µg of protein was diluted to 2 ml with 50 mM potassium phosphate, pH 7.8 containing 0.1% DM. This mixture was combined with 400 µl of liposomes (at 20 mg of lipid/ml), resulting in a 1:40 ratio (wt/wt) of protein to lipids, which was then incubated on ice for 10 min. The mixture was rapidly diluted by mixing with 80 ml of 50 mM potassium phosphate, pH 7.0, on ice. Proteoliposomes were collected by centrifugation at 200,000 × *g* for 2 h at 4°C. Proteoliposomes were frozen (liquid N₂) and then thawed slowly on ice, which was repeated five times.

[¹⁴C]-Neu5Ac Uptake Assay in Whole Cells. For uptake into whole cells we used the BW25113 *ΔnanT* strain from the KO collection (5), which we had modified to create an unmarked, in-frame *ΔnanT* mutant (referred to simply as *ΔnanT*) by using pCP20-mediated removal of the Kan cassette followed by plasmid curing (6). The *ΔnanT* lesion in this strain was confirmed by PCR and sequencing. Aerobic cultures of *ΔnanT* or *ΔnanT* pES7 were grown overnight at 37°C in LB (supplemented with 100 µg/ml ampicillin, where necessary), then diluted 1:100 into fresh 20-ml LB cultures. These were grown aerobically to midlog phase at 37°C, induced with 1 mM IPTG, and allowed to grow for a further 2 h. Cells were harvested by centrifugation at 4430 × *g* for 8 min at 4°C and washed twice with 20 ml of 50 mM KPi, pH 7, and 2 mM MgSO₄. The cells were finally resuspended in the same buffer in a small volume such that the OD₆₆₀ would equal 120. Cells were stored on ice until required.

For uptake assays, 7.6 µl of cell suspension was added to 600 µl of assay buffer to reach an OD₆₆₀ of 1.5. This was incubated at 30°C for 2 min under continuous airflow (water-saturated), at which point 8 µM Neu5Ac (a 50:50 mixture of unlabeled Neu5Ac and [¹⁴C]-Neu5Ac) was added. One hundred-µl samples were taken at 0.5, 1, 2, 3, and 4 min and filtered onto cellulose nitrate membranes with a pore diameter of 0.45 µm (BA85, Schleicher and Schuell). Filters were washed with 2 ml of 50 mM potassium phosphate buffer, pH 7. The cpm (cpm) for each sample was evaluated by using a liquid scintillation analyzer (Packard Tri-carb 2900TR). The assay buffers used were 50 mM KPi, pH 7,

2 mM MgSO₄, and 10 mM D-Li-lactate, which generates $\Delta\mu_H$ and $\Delta\mu_{Li}$; 50 mM KPi, pH 7, 2 mM MgSO₄, and 20 mM NaCl, which generates a $\Delta\mu_{Na}$ and 50 mM KPi, pH 7, 2 mM MgSO₄, and 20 mM LiCl, which generates a $\Delta\mu_{Li}$.

In vivo Cold-Chase Experiments in Whole Cells. For the *in vivo* cold-chase experiments in whole cells, we constructed a $\Delta nanAT$ mutant of *E. coli* unable to take up and metabolize Neu5Ac (this strain will be fully described in Severi *et al.*, manuscript in preparation). Briefly, an unmarked, in-frame $\Delta nanAT$ deletion (consisting of the first six codons of *nanA* fused to the last 12 codons of *nanT*, and flanked at each end by ≈ 500 bp of homologous sequence) was cloned into the temperature-sensitive episomal vector pKO3 (7), and the resulting construct was used to recombine the lesion into the WT *E. coli* strain BW25113 (6) as described in ref. 7. The presence of the correct $\Delta nanAT$ mutation in this strain, which was named SEVY1, was confirmed by PCR and sequencing.

For [¹⁴C]-Neu5Ac uptake assays, single colonies from freshly streaked LB plates of SEVY1 pES1G/pES7 were grown for 6–8 h in LB ampicillin, washed twice in M9 medium, and inoculated at a 1:100 dilution in M9 ampicillin supplemented with 2 mg/ml glucose and 1 mM IPTG. After overnight growth, the cultures were refreshed in the same medium at a starting OD₆₅₀ of 0.1, and grown at 37°C until the OD₆₅₀ reached 0.5, when they were

harvested, washed three times with M9, resuspended to a final OD₆₅₀ of 3 in M9, and stored on ice until use. To assay sialic acid uptake, the cells were diluted 10 times in 4 ml of prewarmed M9, and allowed to acclimatize at 37°C for 2 min with stirring, after which time the assay was initiated by adding [¹⁴C]-Neu5Ac (Sigma) at a final concentration of 1 μ M with continued stirring. The time-course of radiotracer accumulation was followed as described in ref. 8 except that smaller aliquots (200 μ l) were taken. The cold chase was performed by adding 1 mM cold Neu5Ac, or an identical volume of solvent (dH₂O), immediately after taking the aliquot at *t* = 1.5 min. The protein concentration within each sample was determined by a modified Lowry assay (9).

Fluorescence Spectroscopy. Steady-state tryptophan fluorescence spectroscopy was performed by using a Fluoromax 2 spectrofluorimeter (Jobin Yvon) with an excitation wavelength of 295 nm (slit width 5 nm) and an emission wavelength of 328 nm (slit width, 5 nm). The assay mixture consisted of 0.025 μ M VC1779 in 3 ml of 50 mM Tris·HCl, pH 8, and was continuously stirred at 25°C. Increasing concentrations of ligand were added and the ligand induced fluorescence changes were monitored. The dissociation constant (*K_d*) was calculated by plotting the fluorescence change (Δ fluorescence) against ligand concentration (average of four titrations) and fitting a single rectangular hyperbola to the curve by using Sigmaplot.

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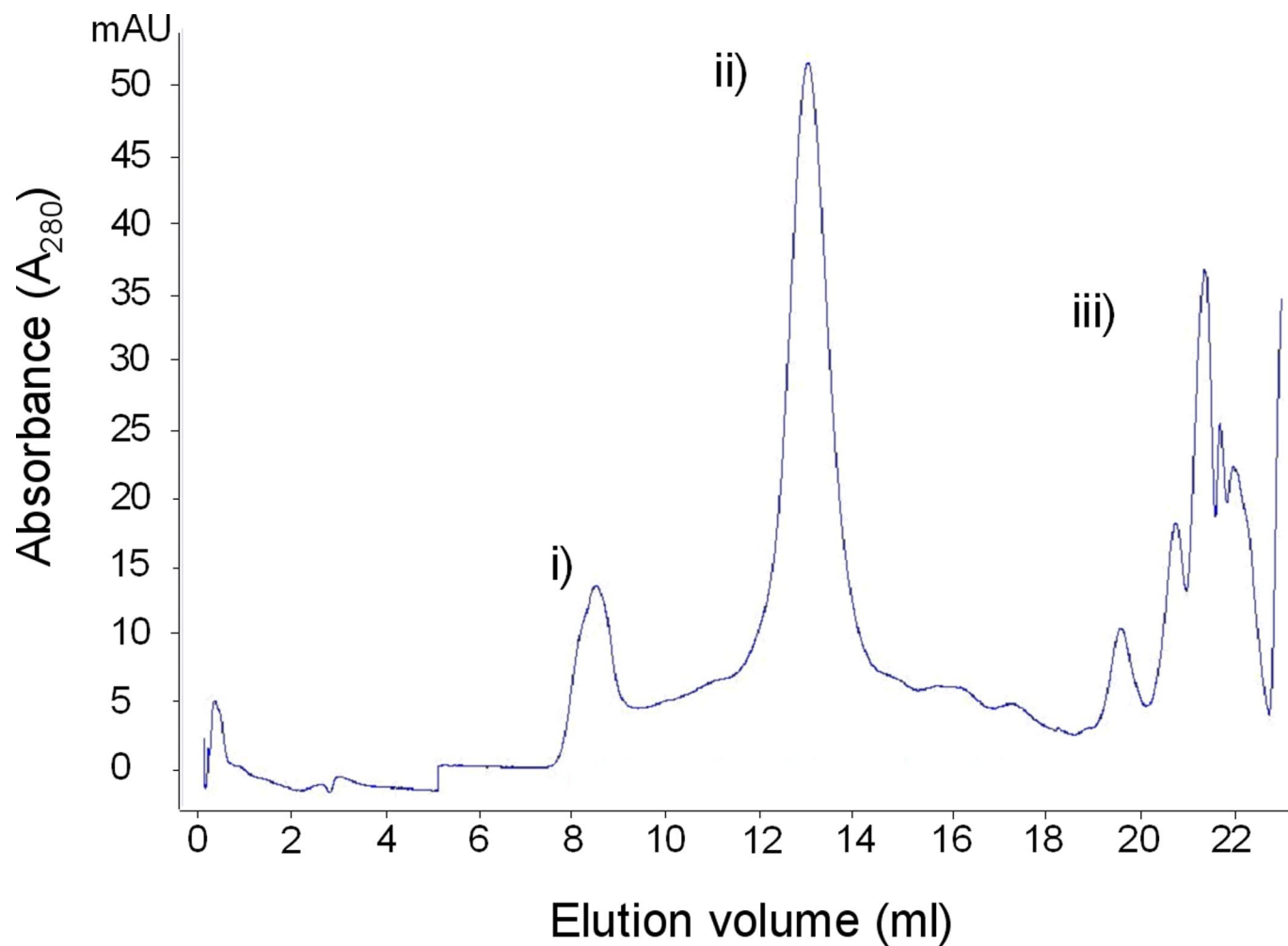


Fig. S1. Analysis of DM-solubilized SiaQM by size-exclusion chromatography (SEC). N-terminally decahistidine-tagged SiaQM purified in DM (as described in the Materials and Methods) was analyzed by SEC with a Superdex 200 column (10/300 GL; Amersham Biosciences) preequilibrated with 50 mM KPi, pH 7.0, 200 mM NaCl, and 0.1% (wt/vol) DM. The absorbance (A_{280}) peaks indicated correspond with; i) aggregates eluting in the void volume, ii) SiaQM, and iii) contaminants that were still present after Ni-affinity chromatography.

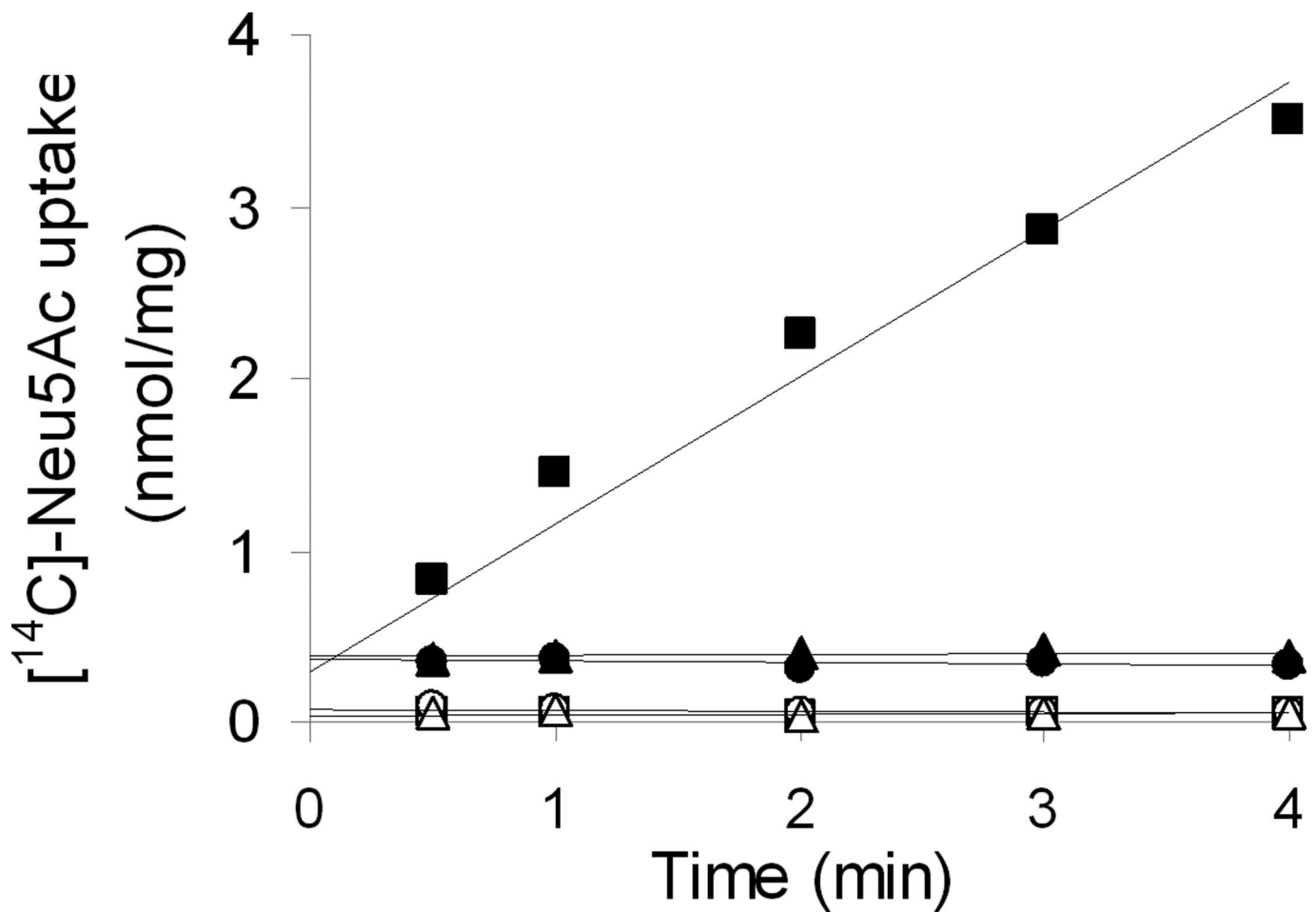


Fig. S2. In vivo Na^+ -driven transport of $[^{14}\text{C}]\text{-Neu5Ac}$ by SiaPQM expressed in *E. coli* $\Delta nanT$. Uptake of $[^{14}\text{C}]\text{-Neu5Ac}$ was monitored into a $\Delta nanT$ strain of *E. coli* in the absence (open symbols) and presence of pES7 (*siaPQM*⁺) (closed symbols) in the presence of no added cations (triangles), 20 mM NaCl (squares) or 20 mM LiCl (circles). The increased background levels in the strains containing pES7 are due to $[^{14}\text{C}]\text{-Neu5Ac}$ bound to periplasmic SiaP.

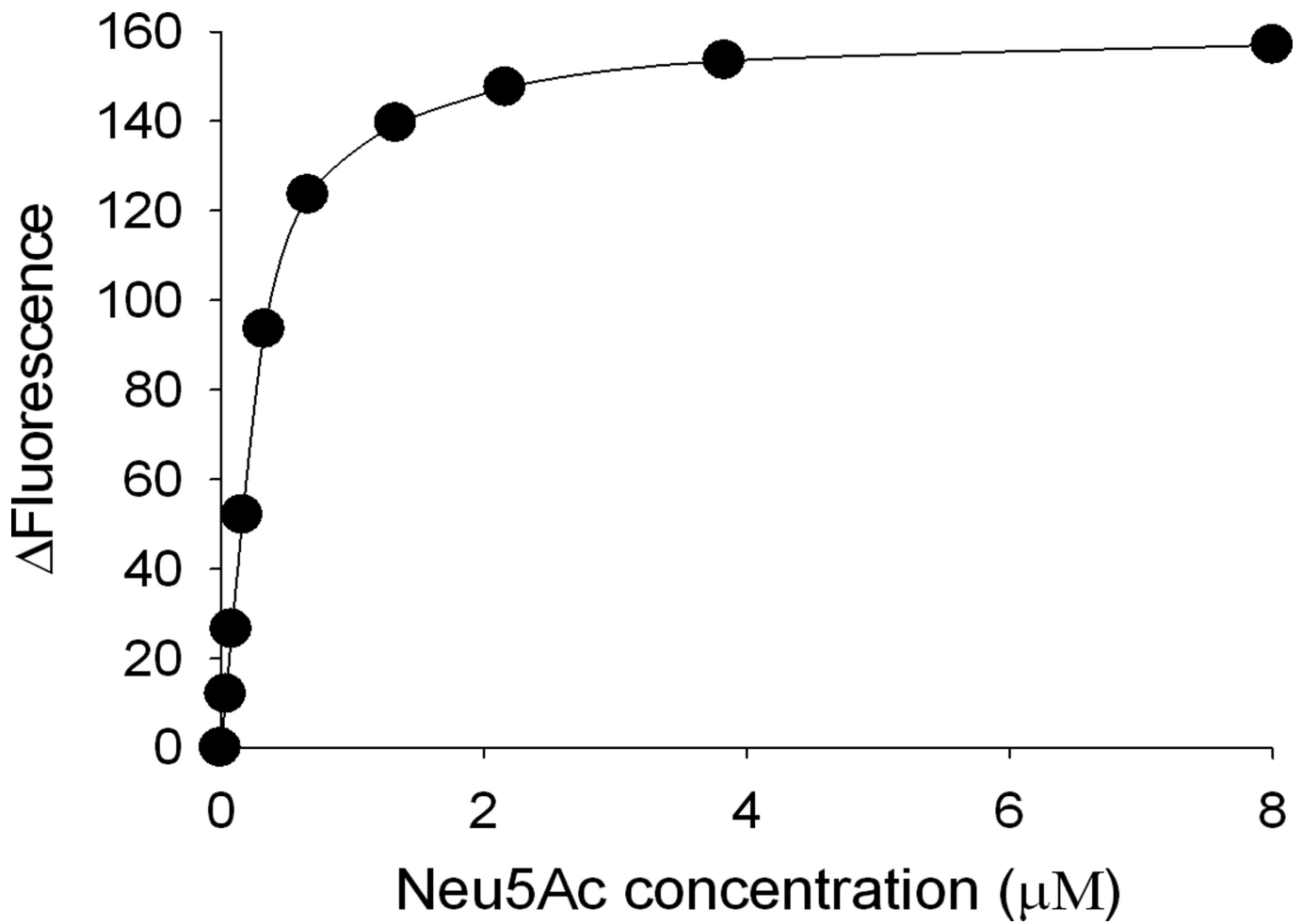


Fig. S3. Titration of VC1779 with Neu5Ac. A protein fluorescence titration was performed with 0.025 μM VC1779 in 50 mM Tris, pH 8, using increasing concentrations of Neu5Ac. The titration was performed with an excitation wavelength of 295 nm and an emission wavelength of 328 nm. The change in fluorescence was plotted against cumulative Neu5Ac concentration and fit to a single rectangular hyperbola. A single representative dataset is depicted.

Name	Sequence (5' to 3')	Gene
SiaP_Nfor	ATGGTGAGAATTTATATTTTCAAGGTATGAAGTTAACAAAACCTTCCCTT	<i>siaP</i>
SiaP_Nrev	TGGGAGGGTGGGATTTTCATTATGGATTAAATTGCTCAATTTGTTTTAA	<i>siaP</i>
SiaQM_Nfor*	ATGGTGAGAATTTATATTTTCAAGGTAAGTATATTAATAAGCTTGAGGAA	<i>siaQM</i>
SiaQM_Cfor*	ATGGGTGGTGGATTTGCTAAATATATTAATAAGCTTGAGGAATGG	<i>siaQM</i>
SiaQM_Nrev	TGGGAGGGTGGGATTTTCATTATGGTATCAATAGATTTGGCACAAATGT	<i>siaQM</i>
SiaQM_Crev	TTGGAAGTATAAATTTTCTGGTATCAATAGATTTGGCACAAATGT	<i>siaQM</i>
pRY11F	CCGCTCGAGCATTTGCTGCCAATTCG	<i>VC1779</i>
pRY11R	CATGCCATGGCGACGACTTTAAAGATGGGG	<i>VC1779</i>
<i>nanT</i> -Asp718G	GCGGTACCGTAAAAGAAGGAGATATACATATGAGTACTACAACCCAG	<i>nanT</i>
<i>nanT</i> -BamHI	GCGGATCCTTAACTTTTGTTTTGACTA	<i>nanT</i>
<i>SiaPQM</i> -Asp718G	GCGGTACCGTAAAAGAAGGAGATATACATATGATGAAATTGACAAAAC	<i>SiaPQM</i>
<i>SiaPQM</i> -BamHI	GCGGATCCTTATGGTATCAATAGATTTGG	<i>siaPQM</i>

*After comparison of the SiaQM sequence to other closely related proteins, it was clear that the original annotation of *H. influenzae* Rd KW-20 used an incorrect start codon. We thus used a later start codon that resulted in a SiaQM protein of 616 aa